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THE METABOLISM OF SULFITE IN LIVER

STIMULATION OF SULFATE CONJUGATION AND EFFECTS ON PARACETAMOL AND ALLYL ALCOHOL TOXICITY

YUEPING SUN, IAN COTGREAVE, BJÖRN LINDEKE* and PETER MOLDÉUS†
Department of Toxicology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden

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Abstract—Sulfite is rapidly oxidized to sulfate in the liver. This was shown both in isolated rat hepatocytes and isolated perfused liver. In addition sulfite treatment resulted in release of GSH originating probably from low molecular disulfides such as GSSG and/or mixed disulfides between GSH and protein sulfhydryl groups. Sulfite was demonstrated to be an efficient precursor for sulfate conjugation. This was demonstrated using paracetamol as a substrate. Sulfite was even more efficient in supplying sulfate for sulfate conjugation than inorganic sulfate. Sulfite was furthermore shown to be protective against the toxicity of both *N*-acetyl-*p*-benzoquinone imine (NAPQI), the reactive paracetamol metabolite, and acrolein, a reactive aldehyde which is a metabolite of allyl alcohol. This protection is most likely due to direct reaction between sulfite and these reactive metabolites in a manner similar to that occurring with GSH and other thiols. When NAPQI and acrolein were generated intracellularly in isolated hepatocytes from paracetamol and allyl alcohol, respectively, toxicity was also expressed. In this case sulfite only protected against allyl alcohol induced toxicity and not against paracetamol induced toxicity. The reason for this discrepancy is not clear but may depend on factors such as site of generation of the reactive metabolite or the reactivity of the reactive metabolite.

The accumulation of inorganic sulfur-containing molecules such as sulfur dioxide (SO_2) and sulfite (SO_3^{2-}) in the biosphere is evoking considerable concern both from environmental and health view points [1]. SO_2 is released into the atmosphere from combustion processes such as those occurring in automobile engines and in coal-fired power stations. This noxious gas dissolves readily in aqueous solutions, such as rain water, yielding various species such as SO_3^{2-} and SO_4^{2-}. Exposure to SO_2 by inhalation is known to produce pulmonary irritation in humans and animals and results in various pathological symptoms in the lungs including perivascular edema and bronchoconstriction [2, 3]. Many of the pathophysiological responses to SO_2 may be mediated through its dissolution products, including SO_3^{2-}. Indeed, SO_3^{2-} is a potent allergen in humans [4] and produces a variety of toxic effects in animals [5, 6]. Sulfite is used extensively as an antioxidant and antimicrobial agent in a variety of chemical, pharmaceutical and food manufacturing industries and is produced during the fermentation of wine and beer [4].

In addition to a potential role as a toxic species in biological systems, SO_3^{2-} may support endogenous biochemical detoxication mechanisms. Firstly, as a substrate for the mitochondrial enzyme sulfite oxidase, which is present in large quantities in the liver [7], SO_3^{2-} may support the sulfate-conjugation of xenobiotics. Secondly, being a strong nucleophile SO_3^{2-} might react directly with reactive electrophilic species which otherwise may react with cellular nucleophilic sites such as the cysteinyl thiol group present in glutathione (GSH) and in proteins (PrSH).

With these activities in mind, it is of interest to note that SO_3^{2-} has recently been shown to be the major systemic metabolite of the thiol drug *N*-acetylcysteine (NAC) when administered intraintestinally in the rat [8]. This drug is administered orally in the treatment of a variety of lung diseases [9] where its mechanism of action is thought to be dependent on extensive intestinal metabolism [8]. NAC is also an effective clinical antidote to paracetamol poisoning, supporting hepatic detoxication of this aminophenol through stimulated GSH biosynthesis in the liver [10].

Despite these studies, little is known of the inter- and intra-organ disposition of SO_3^{2-} in humans or animals. Thus, in view of the central role of the liver in the first pass metabolism of many xenobiotic and natural compounds entering the body via the gastrointestinal tract, we have studied the metabolic disposition of SO_3^{2-} in the isolated, perfused rat liver and in isolated rat hepatocytes. These studies have concerned the kinetics of the metabolism of SO_3^{2-} to inorganic sulfate (SO_4^{2-}) and the effect of SO_3^{2-} on the levels of the endogenous low molecular weight thiol-containing molecules GSH and cysteine (CySH). These studies have centered around the use of recently developed analytical approaches allowing the simultaneous analysis of SO_3^{2-}, GSH and CySH in biological samples [11, 12]. In addition, in view of the potential metabolic and chemical interactions of SO_3^{2-} with endogenous detoxication processes we have further studied the effects of SO_3^{2-} on the metabolism and toxicity of two hepatotoxins, paracetamol and allyl alcohol, in isolated hepatocytes. Paracetamol and allyl alcohol represent a class of xenobiotics causing acute liver toxicity due to their

* Medical department, ACO AB, Box 1827, S-171 26 Solna, Sweden.
† To whom correspondence should be addressed.

"activation" to reactive metabolites. Paracetamol toxicity is thought to be mediated through the generation of *N*-acetyl-*p*-benzoquinone imine (NAPQI) by cytochromes P-450 [13, 14] and allyl alcohol toxicity is thought to result from the generation of acrolein by alcohol dehydrogenase (ADH) [15]. We have also studied the effect of SO_3^{2-} on the metabolism and toxicity of the reactive intermediates themselves in hepatocytes.

MATERIALS AND METHODS

Paracetamol and diethylmaleate (DEM) were obtained from Sigma Chemical Co. (St. Louis, MO). Allyl alcohol (99%) and acrolein (97%) were obtained from Aldrich-Chemie (Steinheim, F.R.G.). *N*-Acetyl-*p*-benzoquinone imine was the kind gift of Dr Sid Nelson, Department of Medicinal Chemistry, University of Washington, Seattle, WA. Materials for the isolation and incubation of rat hepatocytes and isolation and perfusion of rat liver were as referenced. Thiolyte® was obtained from Calbiochem-Behring (La Jolla, CA). All other reagents and chemicals were obtained in the highest grade available from local suppliers.

Animals and treatments

Male Sprague–Dawley rats (200–225 g) were used throughout the experiments and were maintained on a standard laboratory feed and water *ad lib.* for 5 days before use. Livers were removed from animals at the same time of the day following anaesthetization (Mebumal®, ACO Läkemedel, Stockholm, Sweden). Livers were isolated and perfused in a recirculating manner according to the methods of Mehendale *et al.* [16]. Hepatocytes were isolated from animals by the collagenase perfusion method of Moldéus *et al.* [17]. In most cases hepatocytes were prepared from animals maintained on phenobarbital (PB) given at 1% (w/v) in the drinking water for 5 days prior to use. This was performed in order to potentiate the toxicity of paracetamol in the hepatocytes [13, 14]. Additionally, some animals were further treated with DEM 1 hr before hepatocyte preparation in order to deplete hepatocellular GSH.

Perfusions and incubations

Livers were perfused with sulfur-free Krebs–Henseleit buffer pH 7.4 containing HEPES (10 mM) and bovine serum albumin (2% w/v). Additions of SO_3^{2-} to the perfusion medium were made immediately prior to introduction of the organ. Aliquots of perfusate were taken for SO_3^{2-}, GSH, GSSG and SO_4^{2-} analysis as described below.

Hepatocytes were prepared and then washed twice with, and incubated in, sulfur-free Krebs–Henseleit buffer pH 7.4 containing HEPES (10 mM), under an O_2/CO_2 atmosphere. Sulfite, paracetamol, allyl alcohol, acrolein and NAPQI were added to the incubations in various combinations and samples of incubation removed for assay of SO_3^{2-}, GSH, GSSG, SO_4^{2-}, paracetamol and paracetamol metabolites as described below. Cytotoxicity was assessed by the method of trypan blue exclusion.

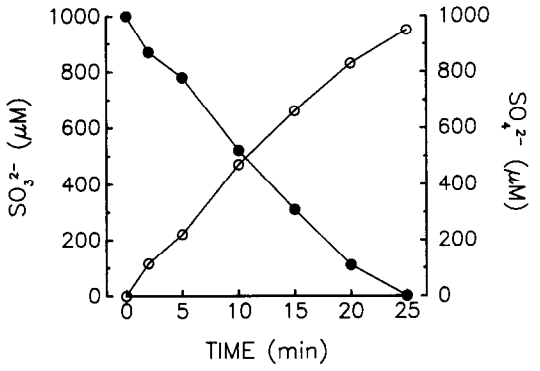

Fig. 1. Metabolism of sulfite (●—●) to sulfate (○—○) in isolated hepatocytes. Incubations were performed at a cell concentration of 1×10^6 cells/ml as described in Materials and Methods. Mean of four experiments, SE < 8% on each point.

Biochemical analysis

Sulfite and free thiols. Sulfite, GSH and CySH were determined in samples by the methods of Cotgreave and coworkers [8, 11]. Briefly, samples were derivatized *in situ* with the thiol-specific, membrane-permeable agent monobromobimane (mBBr). Resultant thiol-mBBr adducts were separated by HPLC and quantitated by fluoresence detection.

Sulfate. Sulfate was determined chemically according to Krijgsheld *et al.* [18].

Oxidized glutathione. Oxidized glutathione was determined enzymatically using the GSSG reductase method of Mize and Langdon [19].

Paracetamol and paracetamol metabolites. Paracetamol and its sulfate-, GSH- and glucuronide-conjugates were determined by their separation on HPLC and quantitation by UV detection essentially according to Moldéus [20]. Reference to authenticated samples of each metabolite was used for peak assignation and calibration.

RESULTS

Sulfite was rapidly converted to SO_4^{2-} by incubation with control rat hepatocytes. The conversion was both linear and quantiative demonstrating a rate of between 35 and 40 μM SO_3^{2-}/min/10^6 cells converted (Fig. 1). This rate was unaltered by either PB or DEM pretreatment of the cells. The rates of conversion of SO_3^{2-} to SO_4^{2-} was similar at initial SO_3^{2-} concentrations of 2 mM, 500 μM and 200 μM (data not shown).

Similarly, perfusion of the isolated rat liver with 1 mM SO_3^{2-} resulted in almost 98% extraction of the SO_3^{2-} during the first 3 min of single pass perfusion. Upon recirculation of the perfusate the remaining SO_3^{2-} continued to decline up to 1 hr (Fig. 2). Concurrently, SO_4^{2-} was shown to rapidly accumulate in the perfusate, reaching 830 μM as quickly as 5 min after beginning perfusion and 930 μM by 30 min, indicating almost a 100% conversion of SO_3^{2-} at this point (Fig. 2). Perfusion of the liver with SO_3^{2-} under these conditions also resulted in the release of GSH in the perfusate in an apparently biphasic manner. By 5 min of perfusion 11.3 μM GSH had rapidly accumulated followed by a slower but steady

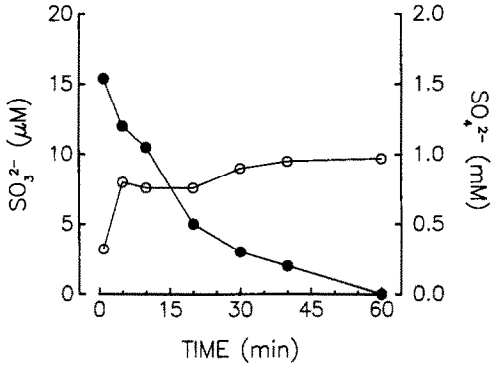

Fig. 2. Metabolism of sulfite to sulfate in the isolated perfused liver. Sulfite (●) and sulfate (○) in the perfusion medium as a function of time. The initial sulfite concentration was 1 mM and the perfusion was recirculating after one pass through the liver. Representative of three experiments.

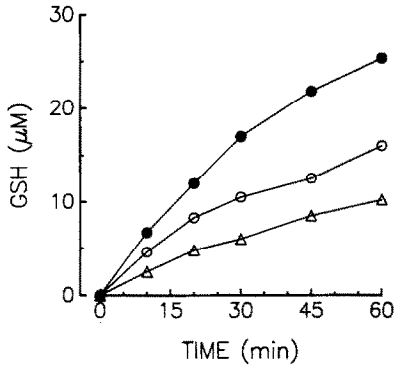

Fig. 4. Reductive cleavage of GSSG to GSH in the presence of sulfite. (△) 50 μM GSSG + 100 μM Na_2SO_3; (○) 50 μM GSSG + 250 μM Na_2SO_3; (●) 50 μM GSSG + 500 μM Na_2SO_3. Reactions were performed in 100 μM phosphate buffer pH 7.4 at 22°.

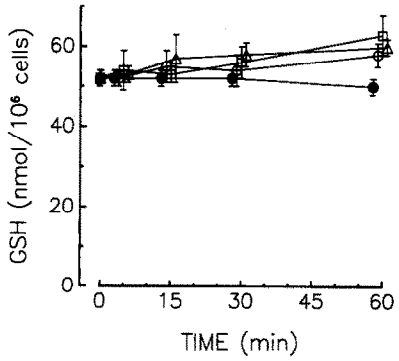

Fig. 3. GSH levels in isolated hepatocytes incubated in the presence of sulfite. (●) control; (○) 0.5 mM Na_2SO_3; (□) 1 mM Na_2SO_3; (△) 2 mM Na_2SO_3. Incubations were performed as described in Materials and Methods. Values are means ± SE of four experiments $P < 0.05$ (between control and Na_2SO_3 treated cells at 60 min).

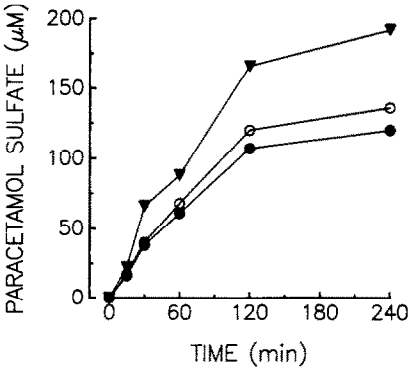

Fig. 5. Paracetamol sulfate conjugate formation in isolated hepatocytes incubated in sulfate free medium. Incubations were performed as described in Materials and Methods at a paracetamol concentration of 2 mM. Representative of three experiments. (●) 500 μM Na_2SO_3; (▼) 1 mM Na_2SO_3; (○) 1 mM Na_2SO_4.

accumulation to 17.2 μM by 60 min. Free cysteine was not detected in the perfusate at any time.

When SO_3^{2-}s effects on isolated hepatocyte GSH and CySH contents were investigated it was shown that incubation of control cells with 0.5, 1 and 2 mM SO_3^{2-} caused a time-dependent increase in the levels of free GSH associated with the cells (Fig. 3). Levels were significantly ($P < 0.05$) elevated after 60 min of incubation with both 1 and 2 mM SO_3^{2-}, reaching 60.6 ± 0.1 nmol/10^6 cells and 62.2 ± 4.8 nmol/10^6 cells, respectively, as compared to control levels of 50.0 ± 3.1 nmol/10^6 cells. When SO_3^{2-} was coincubated chemically with glutathione disulfide (GSSG), a time- and concentration-dependent release of GSH from the disulfide was observed (Fig. 4).

Paracetamol is in the hepatocyte metabolized to glucuronide and sulfate conjugates, in addition to being metabolically activated to a reactive quinone imine by a cytochromes P-450 dependent reaction. As has been shown previously, sulfate conjugation is dependent on an exogenous supply of SO_4^{2-}, as the intracellular concentration of PAPS (adenosine 3′-phosphate 5′-sulfatophosphate) is low [21]. As shown in Fig. 5, SO_3^{2-} was able to efficiently support sulfate conjugation of paracetamol. Control experiments demonstrated that the rate of conversion of SO_3^{2-} to SO_4^{2-} was not affected by the presence of paracetamol in incubations of both cell types (data not shown). Additionally, SO_3^{2-} was more efficient in stimulating paracetamol sulfation than was SO_4^{2-} itself. When 0.5 mM SO_3^{2-} was coincubated with paracetamol the production of paracetamol-sulfate conjugate was equivalent to that produced with 1 mM SO_4^{2-} supplement (Fig. 5).

Sulfite had no effect, however, on glutathione conjugate or glucuronide conjugate formation, nor did sulfite significantly prevent cytotoxicity induced by paracetamol in either control or DEM-pretreated cells (Fig. 6). A protective effect of SO_3^{2-} against cytotoxicity could however be observed if the reactive paracetamol metabolite NAPQI was used to induce cytotoxicity (Fig. 7). When hepatocytes were incubated with NAPQI (250 μM) there was a rapid loss in cell viability with all cells being killed by 30 min of incubation. Similarly, intracellular GSH was totally depleted after only 5 min of incubation. Coincubation of SO_3^{2-} (2 mM) with NAPQI (250 μM) and hepatocytes resulted in considerable protection of both intracellular GSH levels and cell

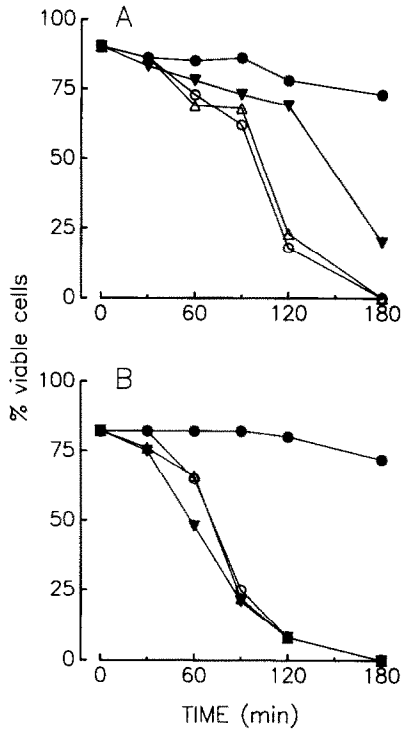

Fig. 6. Effect of sulfite on paracetamol-induced toxicity in isolated hepatocytes. Incubations were performed as described in Materials and Methods using cells isolated from Pb-treated rats and a paracetamol concentration of 5 mM. Representative of three experiments. (A) Normal cells. (B) DEM treated cells. (●) Control; (▼) paracetamol; (○) paracetamol + Na_2SO_3 (2 mM); (△) paracetamol + Na_2SO_3 (2 mM) + 0.5 mM Na_2SO_3 added every 30 min.

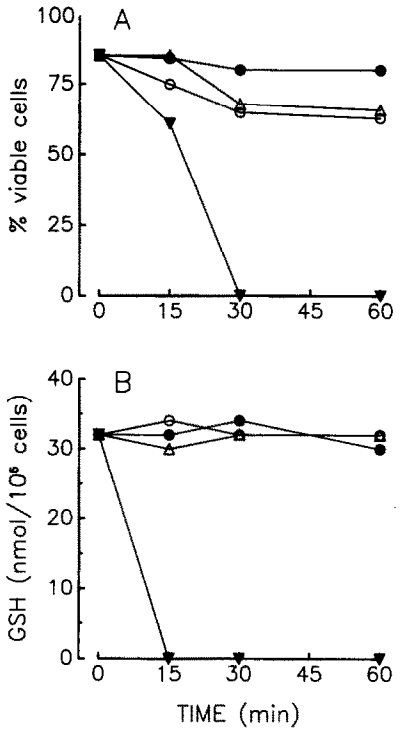

Fig. 7. Effect of sulfite on NAPQI-induced toxicity (A) and GSH loss (B) in isolated hepatocytes. Incubations were performed as described in Materials and Methods using hepatocytes from control rats and a NAPQI concentration of 250 μM. Representative of four experiments. (●) Control; (▼) NAPQI; (△) Na_2SO_3 2 mM; (○) NAPQI + Na_2SO_3.

viability (Fig. 7). Concurrently, analysis of NAPQI metabolites demonstrated that whilst both paracetamol and paracetamol–GSH conjugate were detected in cells treated only with NAPQI the GSH conjugate was not formed in cells cotreated with SO_3^{2-}. A new peak was, however, noted on the chromatogram which accounted for considerable amounts of the added NAPQI. The material in this peak awaits identification.

Similar experiments to the above were conducted with allyl alcohol and its reactive metabolite acrolein. When acrolein (250 μM) was incubated with control cells there was rapid loss of cell viability (100% dead by 60 min) (Fig. 8a) and cellular GSH (100% loss by 15 min) (Fig. 8b). Again, coincubation of cells with acrolein (250 μM) and SO_3^{2-} (2 mM) resulted in an almost total protection of cells from toxicity (Fig. 8a) and substantial protection of the intracellular GSH loss (Fig. 8b). At all time points tested the depletion of SO_3^{2-} in acrolein-treated cells was greater than in control cells treated with SO_3^{2-} alone. This was reflected in lower levels of SO_4^{2-} accumulated in the medium (Fig. 9).

In contrast to results obtained with paracetamol, the toxicity of acrolein's parent compound, allyl alcohol, was greatly diminished by coincubation of cells with SO_3^{2-} (Fig. 10a). Similarly, the allyl alcohol-dependent depletion of cellular GSH was also diminished by coincubation with SO_3^{2-} (Fig. 10b).

DISCUSSION

Inorganic sulfite is rapidly absorbed by isolated rat hepatocytes and isolated, perfused rat liver where it is quantitatively converted to SO_4^{2-} without resultant hepatotoxicity. This hepatic disposition of SO_3^{2-} has several consequences for hepatic metabolism, particularly with regard to cellular detoxication mechanisms involving sulfate conjugation and the tripeptide GSH.

Isolated hepatocytes converted SO_3^{2-} to SO_4^{2-} at a rate of 40 μM/min × 10^6 cells at starting concentrations ranging from 200 μM to 2 mM. The conversion was linear and quantitative over the time period. These results confirm previous observations of the presence of substantial levels of mostly mitochondrially-located sulfite oxidase in liver tissue [7]. Cytotoxicity was not, however, noted with the highest dose of SO_3^{2-} tested (2 mM), which somewhat contradicts previous reports which predict such toxicity in cell types with elevated levels of sulfite oxidase, due to coupling with the electron transport system present in the mitochondria [22].

Under the conditions of perfusion employed, the extraction of SO_3^{2-} from the perfusate by an isolated rat liver preparation followed biphasic kinetics. During the initial phase *ca.* 98% of the added dose (1 mM) was removed in *ca.* 3 min. This was followed by a second, slower decline. The reasons for this biphasic activity are unclear. In the perfused organ, unlike with isolated hepatocytes, SO_3^{2-} must pass

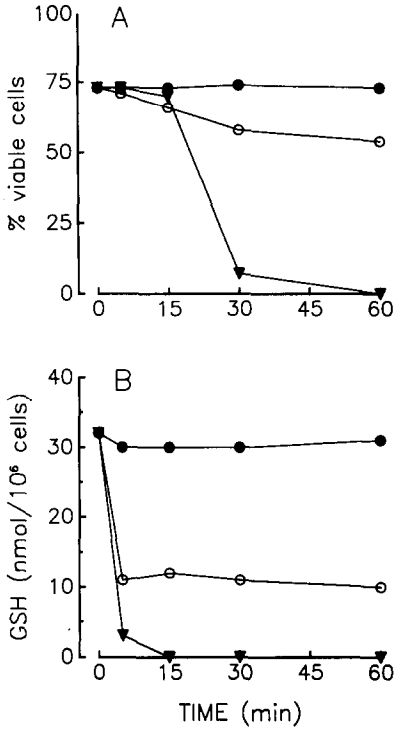

Fig. 8. Effect of sulfite on acrolein-induced toxicity (A) and GSH loss (B) in isolated hepatocytes. Incubations were performed as described in Materials and Methods using 10^6 cells/ml and 250 μM acrolein. Representative of three experiments. (●) Control; (▼) acrolein; (○) acrolein + Na_2SO_3 2 mM.

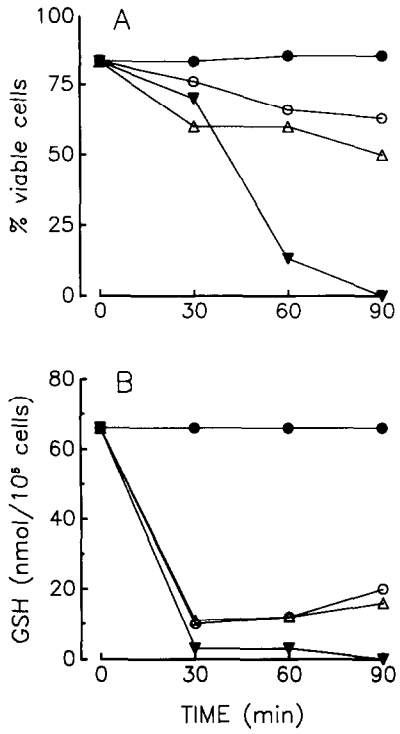

Fig. 10. Effect of sulfite on allyl alcohol-induced toxicity (A) and GSH loss (B) in isolated hepatocytes. Incubations were performed as described in Materials and Methods using 10^6 cells/ml and 500 μM allyl alcohol. Representative of two experiments. (●) Control; (▼) allyl alcohol; (△) allyl alcohol + Na_2SO_3 2 mM; (○) allyl alcohol + Na_2SO_3 2 mM + 0.5 mM Na_2SO_3 added every 30 min.

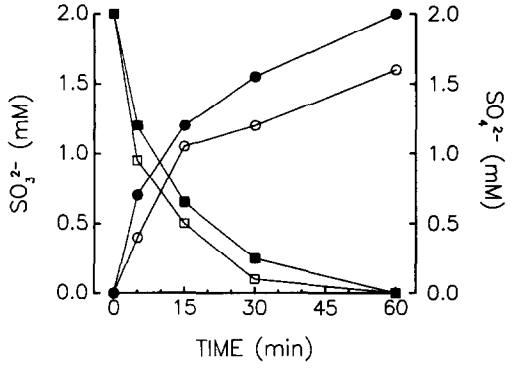

Fig. 9. Sulfite metabolism (□) and sulfate formation (○) in isolated hepatocytes in the presence (open symbols) and absence (filled symbols) of acrolein (250 μM). Mean of three experiments, SE < 10% on each point.

over the endothelial barrier of hepatic blood vessels before being available to the liver cells. It is unknown whether such endothelial cells possess sulfite oxidase. Secondly, the uptake and/or sulfite oxidase-dependent metabolism of SO_3^{2-} may themselves possess biphasic affinities. Although no such biphasic activity was seen with hepatocytes, substrate concentrations in cell experiments ranged from 200 μM to 2 mM whereas the slower phase of extraction of SO_3^{2-} by the perfused organ was noted when SO_3^{2-} concentration was a slow as 20 μM in the perfusate. Although we could not estimate if SO_4^{2-} synthesis paralleled the biphasic decline in SO_3^{2-} as liver SO_4^{2-} was not determined, SO_4^{2-} was rapidly released into the perfusate in concentrations indicating near quantitative conversion, disregarding the dilutory effect of the endogenous SO_4^{2-} pool.

Due to the nucleophilicity of SO_3^{2-} and its close chemical similarity to the organic thiol group, we monitored the behaviour of hepatocellular GSH during the disposition of SO_3^{2-} in these two hepatic model systems. Incubation of hepatocytes with SO_3^{2-} caused a dose-dependent increase in free GSH associated with cells. This is not likely to occur through increased synthesis but by the release of GSH from disulfides in the hepatocytes, which are known to contain GSH mixed disulfides with protein and other low molecular weight thiols and GSSG [11]. Both in the present study and in previous studies SO_3^{2-} was shown to readily perform sulfitolysis of disulfides at physiological pH [23].

Perfusion of the liver with SO_3^{2-} caused an efflux of GSH from the liver into the perfusate. Although it is not certain from these experiments that this is reflected by an elevation of hepatic free GSH, it may be that the efflux originates from mixed disulfides in the liver in an analogous manner to the effect seen in cells. The perfused liver has been reported to release GSH into the perfusate in response to a

number of stimuli including stress hormones such as vasopressin [24]. It is of interest to note here that oral administration of NAC to humans [9] and intraintestinal administration of the drug to rats [8] results in elevation of the systemic levels of free and total GSH in blood plasma. It may be that the considerable first pass intestinal metabolism of NAC to SO_3^{2-} [8] contributes partly to the release of GSH into the circulation by mechanisms similar to those seen in the cell and perfusion experiments detailed here.

As the liver presents one of the main organs where phase I and phase II detoxication reactions occur, we investigated the impact of the hepatic disposition of SO_3^{2-} on the expression of some hepatic detoxication systems. Paracetamol produces acute cytotoxicity in hepatocytes and in the liver *in vivo* through its metabolic activation by cytochromes P-450 to the reaction electrophile NAPQI. When we coincubated NAPQI, SO_3^{2-} and hepatocytes, the cells were protected from NAPQI's toxicity, as well as its depletory effect on intracellular GSH. This protective effect was evident from early time points in the experiments where it is known that SO_3^{2-} exists in considerable excess to NAPQI under the conditions of the experiments. It is probable that the protective effect of SO_3^{2-} was due to direct reactivity with NAPQI. It is well known that other nucleophiles, such as GSH, CySH and NAC, react extremely rapidly with NAPQI to form inert conjugates. On the other hand, SO_3^{2-} has no effect on the toxicity of paracetamol itself in hepatocytes. This may be explained in a number of ways. Firstly, the onset of toxicity of paracetamol in these experiments and others requires considerable time. This is thought to be due to the relatively slow kinetics of generation of NAPQI even in PB-induced cells. Thus, as the oxidation of SO_3^{2-} in the concentrations used is probably complete after *ca.* 1 hr, SO_3^{2-} is not available to conjugate directly with NAPQI.

On the other hand, SO_3^{2-} did not alter the conjugation of paracetamol with either GSH or glucuronic acid. There was, however, substantial support of sulfate conjugation of paracetamol. It has previously been reported that NAC supports the hepatic sulfate conjugation of paracetamol, but that neither basal sulfate conjugation, nor stimulated sulfate conjugation has any effect on the hepatotoxicity of this acetanilide [10].

In contrast to the above situation, SO_3^{2-} coincubation with both acrolein and allyl alcohol protected the cells wholly or partially from their respective toxicities. The cytoprotection against acrolein toxicity by SO_3^{2-} is most probably due to direct reaction between SO_3^{2-} and the molecules aldehydic group in a manner similar to that occurring chemically but not enzymically with GSH [25]. The direct reactivity of SO_3^{2-} is perhaps supported by the observation that acrolein-treated cells always depleted SO_3^{2-} faster and produced SO_4^{2-} slower than did control cells. The protective effect of SO_3^{2-} on allyl alcohol toxicity and cell GSH depletion indicates that SO_3^{2-} competes effectively with cellular GSH for acrolein as it is being generated from the alcohol by alcohol dehydrogenase. The generation rate of acrolein must be sufficient to allow SO_3^{2-} to initiate its protective effects whilst SO_3^{2-} concentrations are still high (i.e. during the first 30 min of incubation).

Taken together, these results indicate that when animals are exposed to sulfite orally, i.e. in the diet, this reactive agent is likely to undergo rapid first-pass metabolic clearance in the liver and may directly support sulfate conjugation of xenobiotics in the liver. Additionally sulfite may indirectly support GSH-dependent detoxication by acting as a competing nucleophile. It is interesting to note also that SO_3^{2-} may be able to react with mixed disulfides between protein thiols and GSH. Many protein activities are thought to be under at least partial control by reversible thiol-disulfide exchange reactions [26, 27]. Indeed, SO_3^{2-} may exert some of its toxicological actions through interfering with such reactions. Such a mechanism may also lie behind the pulmonary effects of oral *N*-acetylcysteine in bronchitis, as SO_3^{2-} is the major first-pass systemic metabolite of the drug [8].

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